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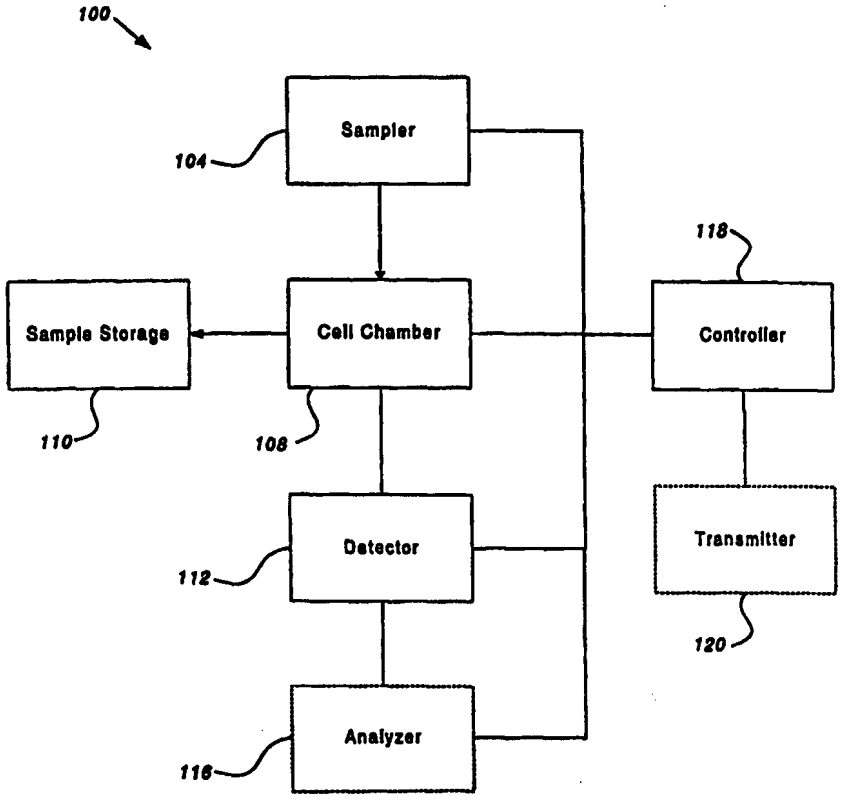
(54) Title: METHOD AND APPARATUS FOR DETECTING HAZARDOUS AGENTS

(57) Abstract

A method and apparatus for determining the presence of a toxic agent, such as a toxic chemical, bacterium, or virus is described. In one embodiment, the apparatus includes a sampler that comprises means for extracting a sample from the environment of the apparatus, such as the ambient atmosphere, water, or soil. Optionally, the sampler can include additional pre-processing means to isolate particular elements of the sample or put the sample in better condition for assay. The apparatus and method can be used to detect a variety of chemical and biological toxins, both natural and non-natural, including chemical and biological toxins of unknown composition and origin.



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METHOD AND APPARATUS FOR DETECTING HAZARDOUS AGENTS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to the detection of biologically active agents. More specifically, the present invention provides methods and apparatus for detecting chemical and biological toxins. The present invention has applications in the fields of epidemiology, environmental safety, counter-terrorism, and chemical/biological warfare.

THE RELATED ART

Humans are exposed to a wide variety of deleterious substances daily. Our natural environment is filled with animals, plants, and microorganisms that produce natural substances that are toxic to humans. For example, many plants and animals produce substances toxic to humans to ward off predators. In addition, the rise of modern industrial economies has provided additional exposure to non-natural toxic substances such as industrial pollutants, pesticides, radiation, and the like. For example, factory workers are exposed to a large variety of toxic emissions from various chemical solvents, coatings, and manufacturing processes. Office workers are exposed to toxic chemical substances by "outgassing" from furniture and office machines. Furthermore, modern technology can interact with natural pathogens to create additional risks. For example, air conditioning units can be efficient breeding grounds for bacteria such *Legionella*, and especially *L. pneumophila*, which are known to cause Legionnaire's Disease, an acute, sometimes fatal, respiratory disease first encountered in 1976.

In some cases the contact between humans and pathogenic organisms and/or toxic chemicals is not incidental. Over the past century a vast technology to utilize various toxic chemical and pathogenic organisms, and specific toxins produced by such pathogenic organisms, in warfare has evolved. The most notorious use of these agents is often associated with the deployment of toxic gases such as mustard gas and phosgene in the First World War, and by the Nazis in their concentration camps during the Second World War. More recently, evidence has been offered that the Iraqi Government used chemical agents to suppress separatist movements following the

Persian Gulf War. The threat posed by biological agents was sufficient that the U.S. and other nations have spent large sums to research both the offensive and defensive uses of biological agents.

The threats posed by the use of chemical and biological warfare ("CBW") agents have become more acute recently in view of the recognition that such agents may be seen by technologically disadvantaged countries or terrorist organizations (both foreign and domestic) as a means to counter advanced military technologies. This reasoning is thought to be especially applicable to toxins produced by pathogenic organisms, as these can be scaled-up and modified (disguised) in an inexpensive manner without the use of large-scale petrochemical or pesticide manufacturing technology. CBW agents also pose a serious threat because of the vast array of unique compounds that can be created.

Unfortunately, technologies for detecting and/or warning of exposure to toxic chemical and biological substances suffer from significant limitations. Electronic devices for detecting chemical agents are limited in sensitivity and range. Systems for detecting biological agents based on arrays of antibodies are also of limited utility, as most human-specific pathogens, such as viruses, are species- or even tissue-specific and therefore produce false negative signals in such detection systems. Furthermore, present detection systems lack significant flexibility with respect to detecting agents whose identities have not been anticipated. Thus, even where detectors have been employed to warn of exposure to toxic agents there is a significant risk of failure where exposure to agents of unknown identity is possible. Although such risks may be greater to military personnel during wartime, exposure to new chemical and biological agents is possible in many circumstances.

Some biosensors detect target molecules either by binding the target to a specific immobilized molecule (*e.g.*, an antibody or receptor directed to the target), or by the interaction between the target and an enzymatic pathway. In the former strategy, binding of the target to the immobilized antibody molecule triggers a change in one of a variety of measured properties (*e.g.*, by measuring changes in surface plasmon resonance, fluorescence, luminescence, mass, or calorimetry). For example, in one antibody-based sensor the binding of a target that is antigenic to a detector

comprising mast cell-bound IgE antibody specific for the target triggers the activation of the mast cells. This leads to the generation of heat, which is registered using a microcalorimiter. In the latter strategy, the presence of a target molecule is detected by its capacity to serve as a substrate for an enzymatic reaction or by virtue of its ability to inhibit a reaction whose progress is being monitored.

Examples of immobilized enzyme-based detection systems include those used for the detection of organophosphates such as the nerve agent Sarin (methylphosphonofluoridic acid 1-methylethyl ester) and VX (methylphosphonothioic acid S-[2-[bis(1-methylethyl)amino]ethyl] O-ethyl ester). Generally, organophosphate nerve agents inhibit the enzyme acetylcholinesterase. Thus, acetylcholinesterase immobilized on a membrane or electrode can be used to generate a signal due to such parameters as the pH change occurring during hydrolysis of its normal substrate or by generation of a current following oxidation of the thiocholine formed by cleavage of butyrylthiocholine. In these cases, presence of an organophosphate is detected by inhibition of the reaction. However, both types of sensor are limited to the detection of known, well-characterized agents for which either an antibody or receptor has been identified and purified.

Recombinant E. *coli* has also been used to detect organophosphates. Cryo-immobilized bacteria engineered to express organophosphate hydrolase were exposed to solutions of organophosphates, and the presence of the compound was detected as a pH change caused by the production of protons during the hydrolysis reaction. Immobilized enzymes, whether present as purified molecules or incorporated into bacteria, allow for a high degree of specificity in detection, provide signal amplification, and are frequently robust. However, the biochemical pathway of action must be known for the agent to be detected. Thus, individual enzyme sensors must be designed for every agent to be detected. Novel agents will therefore escape detection.

Thus, there remains a pressing need for technologies capable of detecting chemical biological agents that are sensitive and robust. The present invention meets these and other needs.

SUMMARY OF THE INVENTION

The present invention provides cell- and tissue-based sensors for detecting chemical or biological agents in which exposure of the sensor to a toxin produces a measurable perturbation in

the function of a normal cell or tissue. Such perturbations include, for example, the loss of electrical activity in a neuron, hyperstimulation of a muscle cell, or disruption of a cellular biochemical process. The devices of the present invention will not only allow the identification of known agents but will also provide advance warning of the presence of novel toxic agents. In addition, such devices can be used to provide a profile of the cellular and gene expression responses to unknown agents; thereby facilitating the development of counter-agents such as vaccines.

In one embodiment, the present invention provides an apparatus for determining the presence of a toxic agent, such a toxic chemical, bacterium, or virus. The apparatus of the invention includes a sampler that comprises a means for extracting a sample from the environment of the apparatus, such as the ambient atmosphere, water, or soil. Optionally, the sampler can include additional pre-processing means to isolate particular elements of the sample or put the sample in better condition for assay.

Samples are passed from the sampler to a cell chamber that includes cells that, when exposed to sample, respond to the presence of toxic agents. The cell chamber is configured to support the growth and maintenance of the cells. The cell chamber further includes detection means to detect and, optionally, quantitate, the response(s) of the cells to any toxic agent(s) in the sample. Such detection can include fluorescence, conductive, and colorimetic detection. According to one embodiment, the cells are derived from mammalian, and, more particularly human, embryonic stem ("ES") or embryonic germ ("EG") cells that have been modified to include one or more elements that produce a detectable, and optionally quantitative, response to toxic insult. In another embodiment, the cells further are further configured to express telomerase and thereby have extended, possibly indefinite, lifespans.

The apparatus further includes a sample storage chamber to store the collected sample for further analysis in addition to data processing means for analyzing data and communications means for transmitting the collected data and/or analysis to remote locations.

These and other aspects and advantages will become apparent when the Description below is read in conjunction with the accompanying Drawings.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates one embodiment of a system for detecting chemical and biological agents in accordance with the present invention.

DESCRIPTION OF SOME EMBODIMENTS OF THE INVENTION

The present invention provides highly specific and robust methods and apparatus for detecting deleterious chemical and biological agents using a variety of cell-based detection techniques. The cells employed in the detectors of the present invention can be any type of human or animal (preferably mammalian, more preferably primate) cell. The use of human or animal cells for detecting chemical or biological toxic agents has the advantage of being less prone to false negative signals, as toxic agents can show species specificity. In some embodiments, the cells used for the detection of dangerous substances include cells from tissues most likely to be exposed to, or be affected by, a toxic agent. For example, those cells most likely to be exposed to an environmental toxic agent include, but are not limited to, bronchial and gastric epithelial cells, neuronal cells, cardiomyocytes, and muscle cells. The cells can be used in a substantially unaltered state, or the cells may include one or more modifications. Examples of such modifications include the inclusion of reporter constructs and lifespan extension by the expression of telomerase. Still other modifications will be apparent to those of skill in the art.

PREPARATION OF CELLS

The cells used in the methods and apparatus of the invention can be obtained from various natural sources, *i.e.*, in a fully differentiated form, or derived from cells of earlier developmental lineage by induced, or uncontrolled, differentiation. In one embodiment, the cells used in the methods and apparatus of the invention are derived from embryonic stem ("ES") or embryonic germ ("EG") cells. ES and EG cells have the advantage of exhibiting normal characteristics (e.g., maintain a normal karyotype through prolonged culture) unlike virally transformed cells, for example. In a more particular embodiment, the ES or EG cells used in the invention are obtained from primate sources (*e.g.*, Rhesus monkey cells). In another more particular embodiment, human ES and EG cells are used. However, it will be appreciated that the source of the ES or EG cells

need only be one effective to provide cells having a morphology suitable for the detection of deleterious environmental agents.

ES AND EG CELLS

ES and EG cells can be obtained from a variety of sources and cultured using known methods and materials. In one embodiment, the ES and EG cells are of primate, more particularly of Rhesus or human, origin. Primate ES and EG cells can be obtained and grown using the methods and materials described in US Patent No. 5,843,780 and in co-pending U.S. Patent Applications Serial Nos. 08/665,217, 08/874,695, 08/829,372, 08/989,744, 08/956,684, 08/961,628, 08/961,629, 08/990,560, 09/052,772, and 09/106,390. Each of these patent applications and patent is incorporated herein in its entirety by reference for all purposes.

DIFFERENTIATION OF ES AND EG CELLS TO FORM SPECIFIC CELL TYPES

ES and EG can be differentiated to form cells of any lineage using a variety of methods. For example, differentiation to specific, desired cells types can be done using methods and materials described in the US patent and co-pending U.S. Patent Applications incorporated by reference above. An example of a broadly applicable method of obtaining substantially pure populations of specific cell types by the differentiation of ES cells includes the use of a cell-type specific promoter effective to drive a selectable marker gene (*e.g.*, one providing resistance to an otherwise toxic drug). Under the appropriate differentiation conditions, in the presence of the drug only those cells that can activate the selectable marker (*i.e.*, those undergoing the desired differentiation) will survive. The surviving cells can then be collected, purified and cultured further to provide a desired quantity of cells (if necessary). Several examples are provided below.

In addition, the cells derived from ES- or EG-cells can include one or more genetic modifications. Such modifications include, but are not limited to, modification of the cells to provide telomerase expression (*e.g.*, using the materials and methods described in Bodnar *et al.*, 1998, as well as those methods and materials described in UK Patent GB2317891 and co-pending U.S. Patent Applications Serial Nos. 08/724,643, 08/844,419, 08/846,017, 08/851,843, 08/854,050, 08/912,951, 08/911,312, 08/915,503, 08/974,584,and 08/974,742, each of which is incorporated in its entirety by reference for all purposes) and/or the inclusion of various reporter

constructs. The modifications can be made using known methods and materials such as described in the above-incorporated, co-pending U.S. Patent Applications.

CARDIOMYOCYTES

In one embodiment, ES- or EG-derived cardiomyocytes are purified further by the use of cardiomyocyte specific promoters driving a selectable marker, *e.g.*, the α-cardiac myosin heavy chain (MHC) promoter fused to the aminoglycoside phosphotransferase (neomycin resistance) gene. Undifferentiated ES cells can be transfected with the α-MHC/neo^r construct, grown as embryoid bodies as detailed above, then plated onto tissue culture dishes in the presence of the drug G418. Under these conditions, essentially pure populations of cardiomyocytes are isolated (Klug *et al.*, 1996). Thus, by transfection of undifferentiated ES cells as described above, large quantities of substantially pure, fully functional cardiomyocytes can be derived.

In addition, distinct subtypes of cardiomyocytes can be isolated based on different patterns of gene expression. For example, atrial (but not ventricular) cardiomyocytes express myosin light chain (MLC) 2a. Thus, applying the techniques described above, atrial cardiomyocytes can be produced preferentially to other subtypes of cardiomyocytes. Conversely, ventricular cardiomyocytes expressing MLC-2v have a pattern of expression complementary to atrial cardiomyocytes and can be cultured by analogy to the methods just described (Klug *et al.*, 1996).

NEUROEPITHELIAL CELLS

In another embodiment, neuroepithelial stem cells are derived from ES or EG cells that are allowed to replicate in insulin-transferin-selenium-fibronectin ("ITSN")-supplemented medium, a medium which is effective in inducing neuronal differentiation in embryonal carcinoma cells (Rizzino and Growley, 1980). These cells are cultured for 6–7 days in the same medium, dissociated, and replated into medium containing basic fibroblast growth factor ("bFGF"). Under these conditions, greater than 80% of the differentiated, proliferating cells express nestin, a marker of the neuroepithelial stem cell (Okabe *et al.*, 1996). Upon removal of the bFGF, neurons, astrocytes, and oligodendrocytes form *in situ*.

The ability to transfect undifferentiated embryonic stem cells also permits a genetic approach to neuroepithelial precursor cell derivation and expansion. As described previously, the use of cell-

type specific promoters driving drug resistance genes allows the selection of specialized cells during ES cell differentiation. Accordingly, if the undifferentiated ES cells are stably transfected with a nestin promoter/neo^r construct, the use of the culture conditions described above combined with drug selection will provide a significant enrichment for neuroepithelial cell precursors.

In addition, genetic approaches allow the potential for greatly expanding the pool of neuroepithelial precursors by intervening in the normal differentiation of the precursors. During normal embryonic development, neuroepithelial precursor cells express a gene denoted Id1 which is repressed upon differentiation (Duncan *et al.*, 1992). This helix-loop-helix protein is a member of a gene family which antagonizes the differentiation of several cell types when overexpressed (Jen *et al.*, 1992; Kreider *et al.*, 1992; Shoji *et al.*, 1994). For example, when neurons derived from mouse embryonal carcinoma cells were forced to express an Id gene, proliferation resumed (Neuman *et al.*, 1995). It has also been demonstrated that expression of the SV40 T antigen (Tag) oncogene allows proliferation of precursor cells, and that normal differentiation can resume upon repression of Tag (Lei *et al.*, 1992; Lew *et al.*, 1993; Alarid *et al.*, 1996). The use of inducible expression systems (*e.g.*, the tetracycline-inducible promoter system) or specific deletion of the overexpression construct through a Cre/lox recombination event allows resumption of the normal differentiation sequence after appropriate expansion of the neuroepithelial precursors.

As described above, if a specific class of neuron is desired, a selectable marker driven by a neurotransmitter-synthetic enzyme promoter can be used to isolate a desired cell type. For example, the tyrosine hydroxylase promoter driving the neo^r gene will select for dopamine-producing neurons over other types of neurons.

HUMAN INTESTINAL EPITHELIAL CELLS.

Normal human intestinal epithelial cells (NHIE) can be obtained from American Type Culture Collection (ATCC, Rockville, MD.). These cells are designated FHs 74 Int and were isolated from a 3–4 month old fetus in 1976 by R. Owens of the Naval Research Laboratory in Oakland, CA (Owens, *et al.*, 1976, Smith, 1979). The cell line retains a normal diploid karyotype, does not form colonies in soft agar, is contact inhibited, and does not induce tumors in immunosuppressed mice (Smith, 1979). The cells have a normal 46 XX karyotype and can undergo a maximum of

approximately 35–40 population doublings in the presence of epidermal growth factor. Normal human intestinal epithelial cells can also be isolated from tissues by established methods. (Owens et al, 1976: Smith, 1979).

HUMAN BRONCHIAL EPITHELIAL CELLS.

Normal human bronchial epithelial cells (NHBE) can be purchased commercially (*e.g.*, from Clonetics Corporation, San Diego, CA). These cells are derived from normal tissue by established techniques. The NHBE grow in a defined medium for a minimum of 15 population doublings. NHBE cells can also be directly isolated from tissues by established methods (Yao, et al. 1997, Lechner *et al.*, 1981, Breza-Squibam *et al.*, 1996, Franklin *et al.*, 1996).

SYSTEMS FOR DETECTING TOXIC CHEMICALS AND BIOLOGICAL AGENTS

In another aspect, the present invention includes systems for detecting toxic substances. One embodiment of such as system is shown at 100 in Figure 1. In the illustrated embodiment, a sampler 104 provides an interface with the environment external to system 100. The sampler comprises one or more means for extracting materials from the environment proximal to system 100. Such means can include means for extracting atmospheric samples, soil samples, water samples. Sampler 104 can further include means for pre-processing samples such as, but not limited to, water and/or organic solvent extractors, heaters, coolers, homogenators, and the like as will be familiar to those of skill in the art.

Sampler 104 is coupled with cell chamber 108. Cell chamber 108 includes cells used for the detection of toxic agents, such as those cell types described above, in addition to systems and materials necessary to maintain the cells in a substantially functional state. Examples of such materials and systems include growth and/or nutrient media, means for removing and adding such growth and/or nutrient media, reagents for performing tests, and means for introducing the sample to the cells (*e.g.*, one or more injectors), as well as means for detecting the response of the cells to the sample. Sample chamber 108 can include only chamber or can comprise several chambers that may, or may not, be hermetically sealed from each other.

In one embodiment, the cells are maintained in tissue culture wells in the multiwell plates including HEPES-buffered tissue culture medium that is maintain at a pH of 7.4. The multiwell

plates are sealed and maintained at 37° C in a mobile tissue culture incubator. The culture system will require a mechanism to periodically exchange media to maintain cell viability. Once placed in the field, air will be sampled at the site and delivered to the multiwells by injection.

In one embodiment, sample materials collected and/or pre-processed by sampler 104 are passed to cell chamber 108 so that the cells contained in cell chamber 108 are exposed to the sample material. Putative toxic agents in the sample material are detected and identified by a series of assays. Toxic agents include, without limitation, chemicals, biologicals and radiation. A first assay detects changes in electrical activity of cell membranes in response to the putative toxic agents. In one embodiment, these cell are the above-described neuronal and cardiomyocyte cells. A second assay monitors fluorescence emissions in a simple live-dead assay to detect cellular lethality. In one embodiment, the cells used for the second assay are the above-described telomerase-transfected human bronchial and intestinal epithelial cells. A final set of assays include the above-described indicator cells to provide information regarding the presence of toxic biological organisms such as bacteria (*e.g.*, anthrax) and viruses (*e.g.*, Ebola or Marburg) in the sample.

Cell chamber 108 is coupled with Sample Storage chamber 110. In one embodiment, upon detection of possible toxic substance in the sample, portions of the sample and the medium bathing the cells in Cell Chamber 108 is taken and stored in Sample Storage Chamber 110 for later recovery and chemical analysis. In another embodiment, the cells are frozen *in situ, e.g.,* using liquid nitrogen in stepwise aliquot fashion over a period of about 12 hours, to facilitate their analysis using gene expression chip arrays as described below, to monitor alterations in gene expression induced by the putative toxic agent. Such a system will allow for the putative toxic agent to be "fingerprinted" and correlated with corresponding chemical analysis of the putative agent from recovered samples and cell medium captured and stored in the field.

Coupled with cell chamber 108 is detector 112 that includes detection means to monitor and, optionally, quantitate the response(s) of the cells in cell chamber 108 to the sample. The detection means used will depend on the type(s) of signal(s) produced by the cells in response to a toxin. Examples of such signals include, but are not limited to, fluorescence-based, conductive, and colorimetric responses. Further illustrations of signals and toxins are provided below. Detector

112 can optionally be coupled with an analyzer 116 that processes signals gathered by detector 112. Such analysis can include waveform and time-series analysis of the signals gathered by detector 112. The analysis can further include pattern recognition to identify pathogenic substances.

The apparatus further includes a controller 118, such as a microprocessor or other multi-purpose computer, to operate the various elements described above. The controller can further be coupled to a transmitter 120 that relays data and/or analysis results to remote locations at which the information so relayed can be further processed or reviewed.

MEMBRANE DEPOLARIZATION

In one embodiment, normal cells obtained from differentiation of primate ES cells as described and telomerase-transfected cells having extended life spans, also prepared as described above, are plated on biosensors typically located at the bottom of a multiwell plate. The biosensors are configured to detect electrical changes in membranes of the cells in response to exposure of the cells to a putative toxic agent. In one embodiment, these changes are recorded as a function of time for further analysis. Such analysis can be performed on-site or at a remote site (following transmission of the recorded data) and can be performed manually (*e.g.*, by reading charts or lists of values including or derived from the recorded data) or with the aid of a machine such as a multipurpose computer.

In one, more particular, embodiment, the plated cells comprise the above-described cardiomyocytes, and the measured electrical activity includes electrical depolarizations associated with each cardiomyocyte contraction. Recorded parameters for this embodiment can include, *e.g.*, the amplitude, frequency, and/or wave form characterizing the electrical behavior of the cells in response to the putative toxin as a function of time. Such data provides a sensitive measure of cardiomyocyte physiology. In other embodiments, neurons are coupled with the biosensor to detect membrane depolarizations, an event associated with toxic insult to such cells. In still other embodiments, the biosensors are coupled with the above-described bronchial and gastric epithelial cells for detection of toxic agents by the live/dead assay as described below.

"LIVE/DEAD" ASSAY

In another embodiment, the detection system of the present invention includes a "live/dead" assay. Such assays distinguish living cells from diseased or dead cells, for example, by their response to cellular uptake of a dye, such as ethidium homodimer ("EthD"). This dye is impermeable to intact cell membranes but permeable once the integrity of the cell membrane is compromised. Ethidium homodimer, free in solution, exhibits low fluorescence; thus, exposure of normal, healthy cells to EthD will provide a low level of fluorescence upon interrogation of the sample. However, when EthD enters cells having compromised membranes (*i.e.*, unhealthy or dead cells) and intercalates with the cellular DNA, the resulting fluorescence is dramatically enhanced. Ethidium homodimer is stable in solution (if protected from light) and is not cytotoxic to the cells and can be added as a standard component to the medium. This assay is simple, rapid and reliable and will give a real-time readout of the effect of the CBW agent on the cells. Such assays are available commercially (Molecular Probes, Eugene, OR).

In a more particular embodiment, an air sample is incubated with telomerase transfected bronchial and/or gastric epithelial cells in a medium containing EthD to expose thereby the cells to the suspected toxic agent. Fluorescence readings are taken periodically using an excitation wavelength of 530 nm and an emission wavelength of 645 nm, (*e.g.*, once every minute) and the resulting data co0llected and analyzed. An increase detection of a "red" signal indicates that the cells' membranes are damaged (*i.e.*, the dye has entered the cells and stained their cellular DNA).

ES/EG DEVELOPMENTAL ASSAY

As described above and in US Patent No. 5,843,780, embryonic stem cells are pluripotent. Embryonic stem cells have the ability to develop into any cell derived from the three germ cell layers or an embryo itself. When injected into SCID mice, an ES cell line will differentiate into the cells derived from all three embryonic germ layers including: bone, cartilage, smooth muscle, striated muscle, and hematopoietic cells (mesoderm); liver, primitive gut and respiratory epithelium (endoderm); neurons, glia cells, hair follicles, and tooth buds (ectoderm). As is apparent to one of ordinary skill in the art, in contrast to a compromised ES cell (i.e., an ES cell affected by a toxic agent), a normal ES cell has the ability to participate in normal development.

Developmental potential can be evaluated by injecting approximately 0.5-1.0 x10^6 primate ES cells into the rear leg muscles of 8-12 week old male SCID mice. The resulting tumors can be fixed in 4% paraformaldehyde and examined histologically after paraffin embedding at 8-16 weeks of development. ES cells injected into the hind leg muscles of SCID mice in this way contribute to normal differentiated tissues derived from all three embryonic germ layers and to germ cells, when analyzed 15 weeks later. An agent having a toxic effect on ES cells can impair ES development and thus toxicity can be evaluated using the technique described above, for example.

DETECTION OF CHOLERA TOXIN

In another embodiment, the presence and identity of toxic agents is detected using cells transfected with a sensitive reporter gene linked to specific promoter sequences. The choice of promoter dictates the stimulus to which the reporter gene responds. Generally, the reporter system is one having the following properties: a) high sensitivity; b) ability to be assayed in living cells; c) utilizes a substrate that is easy to load within cells; and d) is capable of providing real-time responses to the presence of stimuli. In a more particular embodiment, the promoter is one that is activated in the presence of one or more toxins or viruses. In a still more particular embodiment, the cells used in the detection system of the present invention are derived from primate ES or EG cells that have been stably transfected with the reporter gene/promoter sequence just described.

The Gram negative bacterium *Vibrio cholerae* produces cholera toxin, an enterotoxin producing the characteristic massive diarrhea which can cause death within 18 hours of infection. Cholera toxin produces its effects by ADP-ribosylation of a G-protein which leads to the continuous activity of the cellular enzyme adenylate cyclase. The resultant abnormally high intracellular levels of cAMP in intestinal mucosal cells leads to a large efflux of chloride ions into the intestinal lumen, followed by loss of water, sodium cations and other electrolytes.

Thus, in a more particular embodiment, the toxin is cholera and the promoter comprises a cyclic AMP response element ("CRE"). CRE is an octamer consisting of the sequence 5′-TGACGTCA-3′. This sequence can confer cAMP inducibility upon minimal promoters by virtue of its ability to bind members of the cAMP response element binding protein family (Tinti *et al.*,

1996). Promoters which contain cAMP responsive elements (CREs) show large increases in activity when intracellular cAMP levels are raised, either by the direct addition of cAMP to cultured cells or by stimulation of adenylate cyclase by molecules such as cholera toxin or forskolin (Gabellini *et al.*, 1991). Studies on the rat tyrosine hydroxylase promoter have demonstrated that the placement of two CREs within 168 base pairs of the start site was sufficient to confer 4-fold inducibility upon a reporter gene in transient assays when cells were stimulated by forskolin (Tinti *et al.*, 1996). In one embodiment, a vector is provided comprising a reporter gene under the control of a basal promoter (containing a TATA and CAAT sequence) and comprising multiple copies of the canonical CRE within similar distance of the start site, as described above. This construct, when introduced into cells, will serve as an indicator of the presence of cholera toxin, since the reporter gene will be induced by the resulting elevated cAMP. Such cells can be grown on a biologically compatible surface connected to a sensitive detector of the reporter gene product, e.g., fluorescence or chemiluminescence.

In one embodiment, the reporter gene is a LacZ gene. The LacZ gene, encoding E-coli β-galactosidase, has been widely used as a reporter gene in eukaryotic cells. The development of lipophilic fluorescinated-derivatives of di-β-D-galactopyranoside allows sensitive, real-time measurement of β-galactosidase activity in living cells (Zhang *et al.*, 1991). These substrates (commercially available as ImaGene Green and ImaGene Red from Molecular Probes, Inc., Eugene, OR) can be introduced into the normal culture medium, avoiding typical requirements for microinjection or osmotic shock to load the substrate. Prior to cleavage by β-galactosidase, these molecules are non-fluorescent and pass freely through cellular membranes. Once the glycosidic linkage is cut, the product fluoresces and the lipophilic cleavage product is retained within the cell (Zhang *et al.*, 1991). These substrates demonstrate very low toxicity to cultured cells. For example, LacZ-expressing NIH3T3 cells grown for several days in the presence of typical concentrations of these substrates retained normal morphology and continued to divide (Hoagland and Johnson, 1993); NIH3T3 cells and CRE BAG 2 cells grown in medium containing 1mM (20-fold excess) ImaGene Green showed normal morphology and viability (Zhang *et al.*, 1991). These substrates have been used for the measurement of protein production rates during cell cycle

progression in CHO cells (Gu *et al.*, 1993) and for flow cytometric analysis of transgenic mouse sperm (Jasin and Zalamea, 1991). In addition, LacZ-expressing cardiomyocytes derived from murine ES cells showed stable expression of the transgene as detected by fluorescence for over 30 days in culture; these cardiomyocytes showed normal spontaneous contractility (Metzger *et al.*, 1995).

In another embodiment, the reporter used is the E-coli β-lactamase coding sequence in conjunction with a family of membrane-permeable, fluorogenic substrates (Zlokarnik *et al.*, 1998). One such substrate is CCF2/AM a non-polar, non-fluorescent molecule that can penetrate cell membranes. Once inside the cell, nonspecific esterases hydrolyze the molecule's ester groups, leaving the polar CCF2 substrate trapped within the cell. CCF2 contains two distinct fluorphores in close proximity, separated by a target site for β-lactamase cleavage. Due to the overlapping spectra of the two fluorophores, their fluorescence is quenched by fluorescent resonance energy transfer (FRET). Upon cleavage by β-lactamase, the two fluorophores are separated, FRET is disrupted, and the fluorophore remaining attached to the bulk of the substrate molecule (coumarin) glows bright blue (Zlokarnik *et al.*, 1998). The fluorescing cells are viable, thus different timepoints can be measured during the suspected course of exposure to a toxic agent.

IDENTIFYING TOXIC AGENTS BY EXPRESSION PROFILING

An extraordinary range of materials can be classified as toxic agents, including chemicals, biologicals and radiation. Cells have developed many defense and repair systems to protect against such insults and virtually all of these mechanisms include a modification of gene expression. Classical examples of the operation of such systems include responses to heat shock, UV damage, xenobiotic challenge, and viral pathogens. These responses can be monitored by measuring and evaluating changes in gene expression patterns, *e.g.*, by measuring changes in mRNA expression. However, alterations in gene expression patterns have thus far relied on low throughput methods, such as Northern blot analysis and RT-PCR, that typically track only well-characterized genes.

High density DNA arrays have proven to be a powerful means of monitoring mRNA expression patterns. These technologies allow thousands of independent genes to be monitored simultaneously. This is accomplished by depositing or synthesizing target gene DNA onto glass or

silicon surfaces at a density that can surpass 10,000 elements per square centimeter. For example, once the DNA array has been designed and fabricated, the process of expression monitoring begins by converting a population of mRNA molecules into a fluorescent probe which is then hybridized directly to the DNA array. Subsequently, the unhybridized probe is removed by stringent washing and the remaining signal is detected with a fluorescence monitor; the signal at any element of the array is proportional to the relative abundance of an mRNA species in the starting RNA population.

Thus, in one embodiment, a modification of this general procedure is used to perform a comparison of the relative expression levels between two independent RNA populations. The two populations are converted into probe using separate labeling fluors that can be distinguished on the basis of their emission spectra. When the two probes are applied to a single array, they compete for hybridization to the array elements. The relative contribution of the two probes can be quantified, thus defining the relative abundance of a specific mRNA in the two populations. Since much of the interest in monitoring gene expression lies in determining relative changes in expression, as opposed to absolute abundance, this technique has wide application.

In a more particular embodiment, an array comprising at least about 1,000, more particularly at least about 10,000, and, still more particularly, at least about 100,000, independent genes that survey widely the major regulatory systems of the human cell is provided in the above-described detection system. Examples of such genes include, but are not limited to, tumor suppressors, oncogenes, receptors and growth factors, cell cycle components, stress response, and toxicology genes. In another embodiment, the arrays include genes related to additional targets of any origin, such as additional human viral or bacterial genes. In one embodiment, cells prepared as described above are exposed to specific agents (such as viral toxins, xenobiotics and radiation and RNA). The expression patterns of the genes in the cells is determined using the arrays just described arrays and compared to a control. Changes in expression pattern relative to the control are used to determine the presence of a toxic agent.

A clear advantage of using such DNA arrays for monitoring cell responses lies in the high throughput capacity of the technology. Not only can dozens or hundreds of independent

experiments be processed in rapid fashion, each experiment tracks the expression of potentially thousands of independent genes. Such wide-scale monitoring of gene expression reveals obvious and distinctive differences between substantially similar cell types and in the response of a given cell type to related stimuli. These distinctive patterns of expression represent an identifying set of host responses, or gene expression fingerprint, that can be used for monitoring cells when given an unidentified challenge.

EXAMPLE

The following Example is provided to illustrate certain aspects of the present invention and to aid those of skill in the art in practicing the invention. This Example is in not to be considered to limit the scope of the invention in any manner.

IMMORTALIZATION OF HUMAN AND PRIMATE CELLS

Stable transfection of normal human bronchial and intestinal epithelial cells with the catalytic component of human telomerase (hTERT) is carried out according to the procedure of Bodnar, *et. al.* NHBE and NHIE cells are grown in culture and electroporated with either control vector (pBBS212) or vector encoding hTERT with a consensus Kozak sequence downstream of the myeloproliferative sarcoma virus (MPSV) promoter (pGRN145). After 48 hours, transfected cells are placed into medium containing 50 µg/ml Hygromycin-B for 2 to 3 weeks, at which time the concentration is reduced to 10 µg/ml. Individual stable clones are selected and analyzed for telomerase activity by the telomeric repeat amplification protocol (TRAP assay) (Kim, *et al*, 1994).

BIBLIOGRAPHY

The following references are incorporated herein by reference in their entirety and for all purposes.

Alarid, E.T., *et al. Dev.* 122:3319–3329, 1996.

Askanas, V., *et al. Exper. Neuro.* 115:368–375, 1992.

Baeza–Squiban, A., *et al. In Vitro Cell. Dev. Biol.* 27A:453–460 1991.

Bain, G., *et al. Devel. Biol.* 168:342–357, 1995.

Bodnar, A.G., *et al. Science* 279:349–352 1998.

Breckenridge, L.J., *et al. J. Neurosci Res* 42:266–276, 1995.

Bukreyev, A.A., *et al. Biochem. Mol. Biol. Int.* 35:605–613, 1995.

Connolly, Pt., *et al. Biosens. Biores.* 5:223–234, 1990.

Davis, K.J., *et al. Arch. Pathol. Lab. Med.* 121:805–819, 1997.

Dinsmore, J., *et al. Cell Transplant* 5:131–143, 1996.

Doetschman, T.C., *et al. J. Embryol. Exp. Morphol.* 87:27–45, 1985.

Duncan, M., *et al. Dev. Biol.* 154:1–10, 1992.

Dutton, E.K., *et al. J. Neurosci.* 15:7401–7416, 1995.

Finley, M.F.A., *et al. J. Neurosci.* 16:1056–1065, 1996.

Franklin, W.A., *et al. Am J Respir Cell Mol Biol* 15: (3): 297–304 1996.

Gabellini N., *et al.* Exp. Cell res. 194:210–217, 1991.

Gopal, K.V., *et al. Acta Otolaryngol (Stockh)* 116:690–696, 1996a.

Gopal, K.V., *et al. Acta Otolaryngol (Stockh)* 116:697–704, 1996b.

Gordon, C.J. and Watkinson, W.P. *J. Toxicol. Environ. Health* 45:59–73, 1995.

Gordon, D.J., *et al. J. Toxicol. Environ. Health* 45:59–73, 1995.

Gossler, A., *et al. Proc. Natl. Acad. Sci. USA* 83:9065–9069, 1986.

Gu, M.B., *et al. Biotech. Bioeng.* 42:1113–1123, 1993.

Harley, C.B., *Mutation Res* 256:271–282, 1991.

Hayflick, L., *et al.* 25:585–621, 1961.

Hoagand, R.P., *et al. J. Fluores.* 3:119, 1993.

Jahrling, P.B., *et al. Arch Virol. Suppl.* 11:135–140, 1996

Jasin, M., *et al. Proc. Natl. Acad. Sci. USA* 89:10681–10685, 1992.

Jen, Y., *et al. Genes Dev.* 6:1466–1479, 1992.

Kim, N.W., *et al. Science* 266:2011–2015 (1994).

Klug, M.G., *et al. J. Clin. Invest* 98:216–224, 1996.

Kreider, B.L., *et al. Science* 255:1700–1702, 1992.

Lechner, J.F., *et al. Cancer Res* 41:2294–2304 1981.

Lei, K.J., *et al. Mol. Endocrinol.* 6:703–712, 1992.

Lew, D., *et al. Genes Dev.* 7:683–693, 1993.

Maltsev, V.A., *et al. Circ. Res.* 75:233–244, 1994.

Maltsev, V.A., *et al. Mech Dev.* 44:41–50, 1993.

Metzger, J.M., *et al. Circ. Res.* 78:547–552, 1995.

Nakamura. T.M., *et al. Science* 277:911–912, 1997.

Neuman, T., *et al. Exp. Cell Res.* 217:363–367, 1995.

Ohta, T., *et al. Environ. Res.* 77 (1):43-48, 1998

Okabe, S. *et al. Mech. Devel.* 59:89–102, 1996.

Owens, R.B., *et al. J. Natnl Cancer Inst* 56:843–849 1976.

Paddle, B.M. *Biosens. Bioelec.* 11:1079–1113, 1996.

Pizziconi, V.P., *et al. Biosens. Bioelec.* 12:287–299, 1997

Ramanathan, C.S., *et al. Biol. Trace Elem. Res.* 56:93–106, 1997.

Rainina, E.I., *et al. Biosens. Bioelec.* 11:991–1000, 1996.

Rizzino, A. and Growley, C. *Proc. Natl. Acad. Sci. USA* 77:457–461, 1980.

Rohwedel, J., *et al. Dev. Biol.* 164:87–101, 1994.

Sanchez, A., *et al. Virus Res.* 29:215–240, 1993

Schnittler, H.J., *et al. J. Clin. Invest.* 91:1301–1309, 1993.

Shoji, W., *et al. J. Biol. Chem.* 269:5078–5084, 1994.

Smith, H.B. *J Natnl Cancer Inst* 62:225–230 1979.

Thomson, J.A., *et al. Proc. Natl. Acad. Sci. USA* 92:7844–7848, 1995.

Thomson, J.A., *et al. Biol. Reprod.* 55:254–249, 1996.

Veronesi, B., and Ehrich, M. *Toxicol. Appt. Pharmacol.* 120:240–246, 1993.

Wienrich, S.L., *et al. Nat. Genet.* 17:498–502, 1977.

Weintraub, H., *et al. Prodc. Natl. Acad. Sci. USA* 86:5434–5438, 1989.

Wilson, R.J.A., *et al. J. Neurosci Meth.* 53:101–110, 1994.

Wilson, R.J.A., *et al. N. Neurosci Meth.* 53:101–110, 1994.

Wobus, A.M., *et al. Differentiation* 48:173–182, 1991.

Wobus, A.M., *et al. In Vitro Cell. Dev. Biiol.* 30A:425–434, 1994a.

Wobus, A.M., *et al. Roux's Arch. Dev. Biol.* 204:36045, 1994b.

Wyngaarden, J.B., *et al. Cecil Textbook of Medicine*, 1992.

Yao, P.M., *et al. Amer J Physiol* 273:(4, Pt 1): L866–L874 1997.

Zhang, Y., *et al. FASEB J.* 5:3108–3113, 1991.

Zlokarnik, G., *et al. Science* 279:84–88, 1998.

WHAT IS CLAIMED IS:

1. An apparatus for detecting the presence of a toxic agent, said apparatus comprising:

a sampler for receiving a sample;

a cell chamber in fluid connection with said sampler, said cell chamber comprising functional animal cells responsive to the presence of a toxic agent in said sample, and wherein said cell chamber is configured to support maintenance of said cells; and

a means for detecting a response of said cells to the presence of a toxic agent in said sample.

2. The apparatus of claim 1, wherein said toxic agent is a chemical, bacterium, or virus.

3. The apparatus of claim 1, wherein said toxic agent is a bacterial or viral product.

4. The apparatus of claim 1, wherein said sample is an atmospheric sample, a water sample, or a soil sample.

5. The apparatus of claim 1, wherein said sampler further comprises a means to process said sample prior to determining the presence of said toxic agent in said sample.

6. The apparatus of claim 1, wherein said cell chamber further comprises a means for quantifying said response.

7. The apparatus of claim 1, wherein said cell response detecting means is by fluorescence, conductive, or colorimetic means.

8. The apparatus of claim 1, wherein said cells are embryonic stem cells or embryonic germ cells.

9. The apparatus of claim 1, wherein said cells are human cells.

10. The apparatus of claim 1, wherein said cells are cardiomyocytes, neuroepithelial cells, intestinal epithelial cells or bronchial epithelial cells.

11. The apparatus of claim 1, wherein said cells express telomerase.

12. The apparatus of claim 1, further comprising a sample storage chamber in fluid connection with said sampler.

13. The apparatus of claim 1, further comprising a data processing means linked to said detecting means.

14. A method for detecting the presence of a toxic agent in a sample, said method comprising the use of the apparatus of claim 1.

15. A method for detecting the presence of a toxic agent in a sample, said method comprising:

exposing functional animal cells to a sample potentially comprising a toxic agent; and

detecting any toxic response of said cells.

16. The method of claim 15, wherein said animal cells are human cells.

17. The method of claim 15, wherein said animal cells are bronchial epithelial cells, gastric epithelial cells, neuronal cells, cardiomyocytes or muscle cells.

18. The method of claim 15, wherein said animal cells are genetically modified.

19. The method of claim 15, wherein said animal cells express telomerase.

20. The method of claim 15, wherein said animal cells are or are derived from primate embryonic stem cells or embryonic germ cells.

21. The method of claim 20, wherein said animal cells are human cells.

22. The method of claim 15, wherein said sample is an atmospheric sample, a soil sample or water sample.

23. The method of claim 15, wherein said toxic response is detected by changes in electrical activity of the cell membrane.

24. The method of claim 15, wherein said toxic response is detected by fluorescence emission.

25. The method of claim 15, wherein said toxic response is detected using a reporter gene construct.

26. The method of claim 15, wherein only a portion of said sample is exposed to said cells and the remaining sample is stored.

27. The method of claim 15, wherein said cells are frozen after exposure to said sample.

28. The method of claim 15, wherein said toxic response is quantified.

29. The method of claim 15, wherein said toxic response is detected by fluorescence-based, conductive or colorimetric methods.

30. The method of claim 29, wherein said toxic response is detected by fluorescent resonance energy transfer.

31. The method of claim 15, wherein said toxic response is detected by determining alterations in gene expression in said cells relative to cells not exposed to said sample.

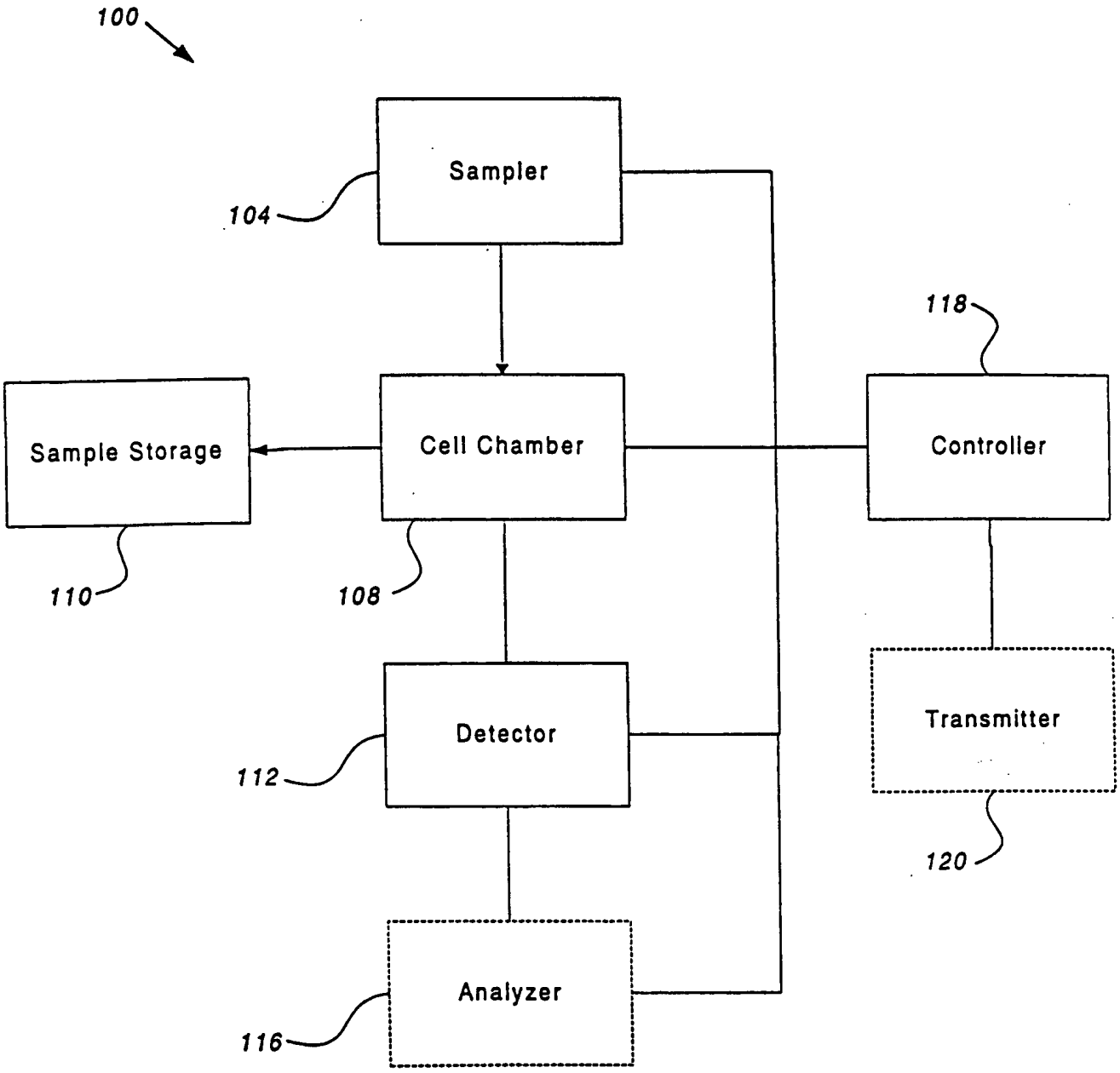
32. A method of classifying a putative toxic agent, said method comprising:

(a) exposing functional animal cells to said potential toxic agent;

(b) determining alterations in gene expression for said cells compared to untreated cells.

33. The method of claim 32, wherein said alteration in gene expression is identified as identical to or substantially the same as that obtained with a known agent, thereby identifying said putative toxic agent as said known agent.

FIGURE 1


100
Sampler
104
118
Sample Storage
Cell Chamber
Controller
110
108
Detector
Transmitter
112
120
Analyzer
116